Brain Research Bulletin, Vol. 51, No. 4, pp. 345–355, 2000

0361-9230/00/$–see front matter

ELSEVIER

PII S0361-9230(99)00246-4

Sodium overload through voltage-dependent Na^+ channels induces necrosis and apoptosis of rat superior cervical ganglion cells *in vitro*

T. Koike,[1]* S. Tanaka,[1] T. Oda[1†] and T. Ninomiya[2]

[1]*Molecular Neurobiology Laboratory, Graduate School of Science, Hokkaido University, Sapporo, Japan; and*
[2]*Department of Anatomy, School of Medicine, Sapporo Medical University, Sapporo, Japan*

[Received 22 March 1999; Revised 25 August 1999; Accepted 11 October 1999]

ABSTRACT: Using the failure to exclude trypan blue as a criterion for cell death, we found that veratridine, the voltage-dependent Na^+ channel activator, exerted its toxicity to cultured sympathetic neurons in a dose-dependent manner (half-maximal toxicity occurred at 2 μM). The co-presence of tetrodotoxin completely reversed the toxicity only at concentrations of veratridine <20μM. Veratridine neurotoxicity was due to the influx of Na^+; a medium low in Na^+ (36 mM) completely abolished its neurotoxicity, whereas a Ca^{2+}-free medium did not attenuate its neurotoxicity. Furthermore, the buffering action of 1,2-Bis-(2-aminophenoxy)ethane-N,N,N′,N′,-tetraacetate (BAPTA) on veratridine-induced increase in intracellular Ca^{2+} levels neither blocked veratridine neurotoxicity in normal medium, nor attenuated the low Na^+ effect. Elevated K^+ effectively blocked veratridine neurotoxicity in a Ca^{2+}-dependent manner. Cytoplasmic pH measurements using a fluorescent pH indicator demonstrated that cellular acidification (from pH 7.0 to pH 6.5) occurred upon treatment with veratridine. Both veratridine-induced acidification and cell death were ameliorated by 5-(N-ethyl-N-isopropyl)amiloride, the specific inhibitor of the Na^+/H^+ exchanger (IC_{50} = 0.5 μM). Finally, necrosis occurred predominantly in veratridine neurotoxicity, but both staining with bisbenzimide and TUNEL analysis showed nuclear features of apoptosis in sympathetic neurons undergoing cell death.

KEY WORDS: Cytoplasmic acidification, Na^+/H^+ exchanger, Sympathetic neuron, Veratridine, TUNEL, NGF, Calcium.

INTRODUCTION

The generation of electrical activity associated with the development of voltage-dependent Na^+ channels is a signal for neuronal differentiation and maturation [34] and is a key element in the regulation of neuronal survival and/or cell death [19,20]. Pharmacological intervention of presynaptic transmission [24] or deafferentation [11] results in enhanced neuronal death in sympathetic ganglia during development. In contrast, veratridine or veratrin, the activator of voltage-dependent Na^+ channels, promotes the survival of sympathetic neurons dissociated from the chick embryo [5] or delays the cell death of rat sympathetic neurons following nerve growth factor (NGF) deprivation [36]. Upon maturation, neurons become vulnerable to hyperexcitability, which often causes cell damage and death through the breakdown of cellular ion homeostasis in neurons from the central nervous system. Thus, veratridine induces the cell death of cortical neurons [22,25,31], hippocampal neurons [3,26,28,29], and cerebellar granule neurons [4,8] *in vitro*. Veratridine neurotoxicity is ameliorated by tetrodotoxin (TTX) or flunarizine, suggesting the involvement of voltage-dependent Na^+ channels [3,28,29]. There is evidence that the binding of veratridine to a specific site of voltage-dependent Na^+ channels allow not only Na^+, but also Ca^{2+} to enter into the cytoplasm through these channels in neuroblastoma cells [18]. Membrane depolarization through Na^+ entry via voltage-dependent Na^+ channels may activate voltage-dependent Ca^{2+} channels, which then allow the entry of extracellular Ca^{2+} into the cytoplasm. Alternatively, the Na^+/Ca^{2+} exchanger that maintains neuronal Ca^{2+} homeostasis may operate in a reverse fashion under conditions where cytoplasmic Na^+ levels are elevated in the presence of veratridine [28,29]. In any case, the massive influx of extracellular Ca^{2+} is likely. During the last decade, the role of cytoplasmic calcium overload has been drawn a major focus of investigation as a possible cause of veratridine neurotoxicity as well as excitotoxicity. The possibility of the direct involvement of Na^+, independently of Ca^{2+}, remains to be examined under various pathological conditions, such as brain injury in ischemia or anoxia or other insults to the brain [9,10,15,22,27,32,39]. We have examined the role of Na^+ in veratridine neurotoxicity in sympathetic neurons.

Superior cervical ganglion (SCG) cells are dependent on NGF for survival, and their cell death has been extensively studied [19]. These neurons do not express glutamate receptors on their cell surface, thus allowing us to analyze the detailed mechanism of veratridine action without secondary excitotoxic effects. There is ample evidence from the sympathetic neurons suggesting that depolarizing signals may exert their effects on neuronal survival through a Ca^{2+}-dependent mechanism [20]. Chronic depolariza-

* Address for correspondence: Dr. T. Koike, Molecular Neurobiology Laboratory, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan. Fax: +81-11-706-4448; E-mail: tatsurok@ccms1.hucc.hokudai.ac.jp
† Present address: Abiko Research Laboratory, Central Research Institute of Electric Power Industry, Chiba 270-1166, Japan.

tion with elevated K^+, which mimics some aspects of naturally occurring electrical activity, has proved to support neuronal survival via L-type Ca^{2+} channels in a variety of cell types [20]. Measurements of intracellular free Ca^{2+} levels ($[Ca^{2+}]i$) in neurons loaded with fura-2, suggest the possibility that neuronal survival is associated with an optimal level of $[Ca^{2+}]i$ and will not be maintained when the level goes beyond or below an optimal level [7,21]. It is not known whether or not the activation of voltage-dependent Na^+ channels affect cell survival and/or cell death in a similar manner.

Neuronal cell death is divided into two categories, apoptosis and necrosis primarily based on biochemical and morphological criteria including chromatin condensation and fragmentation occurred in neurons undergoing cell death. There is ample evidence suggesting that hypoxic-ischemic injury causes a mixed response of early necrosis and delayed cell death characterized by apoptosis [6]. Excitotoxicity due to overstimulation of glutamate receptors often causes necrosis associated with apoptosis under certain circumstances [2,30,33]. Veratridine-induced cell death is reported to occur without DNA laddering in cerebellar granule neurons [8]. A number of factors, however, including the intensity of the insult, the cell type, and the developmental stage of the neurons should be taken into consideration for distinguishing the mechanism of cell death. Although there is evidence that intracellular Na^+ entry may potentiate hypoxic-ischemic cell death by causing cytotoxic cell edema, intracellular acidosis, and gating of Ca^{2+} entry [1], the major pathway of cell death remains elusive.

Here, we provide evidence that veratridine neurotoxicity occurs through Na^+ overload and the subsequent involvement of the Na^+/H^+ exchanger that results in cytoplasmic acidification and eventually cell death in SCG neurons. Necrotic cell death was predominant, but apoptotic features of cell death were also observed in SCG neurons undergoing cell death.

MATERIALS AND METHODS

Cell Culture

Dissociated SCG neurons were prepared from newborn Sprague–Dawley rats as described previously [35,36]. Briefly, dissected ganglia were treated with 1.0 mg/ml collagenase at 37°C for 60 min, washed, and then suspended in Eagle's minimum essential medium (MEM; GIBCO BRL Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA). The ganglia were triturated, and the cell suspension was filtered through a nylon mesh. The dissociated neurons were plated onto a center area of collagen-coated plastic plates (48-well dish, 0.2 ganglia/well) for cytological and drug experiments or onto glass coverslips (12 mm diameter or 25 mm diameter, 0.17 mm thickness, 0.2 ganglia/plate) for immunohistochemical or fluorescence imaging experiments. The neurons were grown in a fresh 10% FBS/MEM medium supplemented with 50 ng/ml NGF for 6–7 days at 36.5°C in a humidified atmosphere of 5% CO_2/ 95% air. Fluorodeoxyuridine (15 μM) was added together with 15 μM uridine to the feeding medium to suppress the growth of non-neuronal cells.

Evaluation of Cell Death of Cultured SCG Neurons

SCG neurons having been exposed to NGF for 6–7 days were further incubated for 15–24 h in the medium supplemented with NGF in the absence or presence of the indicated concentrations of veratridine. The viability of these neurons was evaluated by dye extrusion with 0.2% trypan blue [36] or by measurements of adenylate kinase released into the medium [20]. Both methods gave essentially the same results. Only the data obtained from the dye extrusion method are presented. Control and drug-treated neurons were incubated at 36.5°C for 1 h by adding an equal amount (0.2 ml for 48 wells) of 0.4% trypan blue in 0.12 M Na^+-phosphate buffer, pH 7.2. The neurons were then washed once with Dulbecco and Vogt's phosphate buffered saline (PBS), pH 7.2 followed by fixation with 4% paraformaldehyde (PFA)/ 0.12 M Na^+-phosphate buffer. Neuronal death is shown as a percentage of the number of trypan blue-stained neurons out of the total neurons counted per area (center of each well, 0.65 mm × 0.45 mm, more than 500 cells). Each point represents an average of values ± SEM obtained from three different wells. Experiments were performed at least three times, and representative results are shown.

When necessary, the concentration of Na^+ was replaced with 142 mM N-methyl-D-glucamine instead of NaCl. In the case of the Ca^{2+}-free medium, the serum was extensively dialyzed against Ca^{2+}-free MEM for 3 days. The neurons were washed twice with this Ca^{2+}-free medium containing 10% dialyzed serum and 0.2 mM EGTA prior to testing the effect of veratridine in the Ca^{2+} free medium. The neurons were viable for at least 3 days in this Ca^{2+}-free medium in the presence of NGF.

Measurements of Intercellular Free Ca^{2+} ($[Ca^{2+}]i$), Na^+ ($[Na^+]i$), or H^+(pH)

Fluorescence Ca^{2+} Imaging (Hamamatsu Photonics ARGUS 50) was employed for measurements of $[Ca^{2+}]i$ levels of fura-2-AM loaded neurons as described in detail previously [21], except that the neurons were loaded with 2μM fura-2-AM for 1 h. Fluorescence measurements were performed on single neurons and small aggregates to collect fluorescence signals from individual neurons in HEPES saline, pH 7.2, maintained at 32–34°C. A calcium calibration buffer kit I from Molecular Probes, Inc. (Eugene, OR, USA) was used for the conversion from the measured values of fluorescence ratios to the concentrations of Ca^{2+} [21,38]. The levels of $[Na^+]i$ were measured similarly by use of the Na^+-sensitive dye, sodium-binding benzofuran isophthalate-acetxymethyl ester (SBFI-AM) [16,36]. The neurons were loaded with 10 μM SBFI-AM for 2 h in 10% FBS/MEM supplemented with Pluronic F-127 [16]. Calibration of the fluorescence ratio (340/380) in terms of $[Na^+]i$ was done *in situ* by applying 2 μM gramicidin D, 0.5 μM monensin, and 5 μM nigericin to the neurons as described [16,36].

SCG neurons were loaded with 2μM 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxylfluorescein acetoxymethylester (BCECF-AM, Dojindo) for 1 h, and then cytoplasmic pH were measured in HEPES saline, pH 7.2 in the presence of 26 mM sodium bicarbonate. Calibration of the fluorescence ratio (490/450) into pH was done *in situ* by exposing the neurons to 15 mM HEPES-MES buffer with various pHs containing 130 mM KCl, 1 mM $MgCl_2$ in the presence of 5 μM nigericin [37].

Detection of DNA Fragmentation

The TUNEL assay was performed using Boehringer Mannheim kit according to the manufacturer's procedure, except the treatment with the primary antibody for 1 h at room temperature. The cells were also stained with 1 μM bis-benzimide (Hoechst 33258; Wako Biochemicals, Tokyo, Japan) in PBS for 15 min, and then visualized under UV illumination (Nikon Diaphoto 300). Apoptotic nuclei were identified as those with chromatin formation of discrete chromatin clumps for bis-benzimide-staining and those with stained with the TUNEL. The results are shown as a percentage of the number of neurons stained with bis-benzimide or the TUNEL out of that of total neurons (more than 300 cells/well), and is the mean ± SEM (n = 3). Experiments were performed three times, and representative results are shown.

Transmission Electron Microscopy

SCG neurons grown in collagen-coated 35-mm plastic dishes were fixed with 2.5% glutaraldehyde in PBS, and then stained with bis-benzimide as described. The neurons with chromatin undergoing DNA fragmentation or with normal chromatin were identified by marking the area of plastic plates to which the identified neurons were attached. The neurons were then post-fixed with 1% osmium tetraoxide. They were dehydrated through a graded alcohol series and embedded in Epon 812. Ultra-thin sections of the identified regions were cut on a Reihert-Nissei ultra cut microtome with a diamond knife and then stained with uranyl acetate and lead citrate. The sections were examined under a Nihondenshi electron microscope.

Materials

Mouse NGF (2.5 S) was isolated from male adult mouse submaxillary glands as described [21]. Alpha-scorpion toxin ScTX) was a gift of Dr. Miyake, Hokkaido University. Tetrodotoxin (TTX) and benzamil were purchased from ICN Biochemicals, Inc. (Irvine, CA, USA) and 5-N-ethyl-N-isopropyl-amiloride (EIPA) was from RBI (Natick, MA, USA). Veratridine and flunarizine were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fura-2-AM, SBFI-AM, BCECF-AM, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetate acetomethoxyester (BAPTA-AM), and Pluronic F-127 were obtained from Molecular Probes, Inc. or from Dojindo Chemicals Co. Ltd (Kumamoto, Japan). All other reagents were of reagent quality.

RESULTS

Veratridine Induces Cell Death in Rat Sympathetic Neurons In vitro

SCG cells dissociated from newborn rat pups were grown for 6–7 days in the presence of NGF. Using the failure to exclude trypan blue as a criterion for cell death, we found that veratridine, the voltage-dependent Na^+ channel activator, exerted its toxicity in a dose-dependent manner (half-maximal neurotoxicity occurred at 2μM) to these mature neurons with somatic hypertrophy and extensive neurites (Figs. 1A,B, Fig. 2). Figure 1B shows that cell bema became dark and shrunken under the phase-contrast microscope and neurites were completely fragmented. The co-presence of 1 μM TTX completely rescued the neurons from cell death, suggesting that veratridine neurotoxicity occurred through voltage-dependent Na^+ channels (Fig. 1C, Fig. 2). It should be noted, however, that such specificity was lost with concentrations of veratridine >20μM under our conditions (Fig. 2). In the following experiments, we employed 5–10 μM veratridine in order to maintain the specificity of action.

Measurements of Levels of $[Na^+]i$ and $[Ca^{2+}]i$ in Cultured SCG Neurons in Response to Veratridine

The binding of veratridine to a specific site in voltage-dependent Na^+ channels enhances their open state probability. It is well known that higher concentrations of veratridine induce an increase of not only Na^+ but also Ca^{2+} influx through its binding to voltage-dependent Na^+ channels in neuroblastoma cells [18]. SCG neurons incubated for 6–7 days in the presence of NGF were loaded with SBFI-AM or fura-2-AM, and the effects of veratridine on the levels of $[Na^+]i$ or $[Ca^{2+}]i$, respectively, were examined. Figure 3B shows that treatment with 10 μM veratridine resulted in a sustained increase in cytoplasmic Ca^{2+}; treatment with 1 μM TTX prior to veratridine application completely eliminated this response (Fig. 3C). In contrast, $[Na^+]i$ levels of veratridine-treated neurons gradually increased (Fig. 3D); the basal value of $[Na^+]i$ (4.2 $\pm$ 2.7 mM) increased to 40 $\pm$ 22 mM when neurons were treated with 10 μM veratridine for 60 min (mean $\pm$ SD, n = 28; Student's t-test, $p < 0.005$). Veratridine-induced increase in $[Na^+]i$ was completely suppressed by the presence of TTX (1 μM; Fig. 3E). Veratridine-induced increase in $[Na^+]i$ was also blocked by flunarizine (1 μM) (data not shown) which also suppressed veratridine neurotoxicity (half-maximal effect occurred at 0.25 μM). The dose-response curve of the level of $[Na^+]i$ as a function of veratridine concentration shown in the inset of Fig. 2 indicates $EC_{50} \approx 3$ μM, which roughly coincides with that of neuronal cell death (EC_{50} = 2 μM in Fig. 2).

Alpha-scorpio toxin (ScTx) is also known to stimulate the influx of Na^+ and neurotoxic in cerebellar granule neurons [8]. We found that this toxin was not toxic in SCG neurons at the concentrations examined (up to 5 μg/ml). However, ScTX (1 μg/ml) potentiated the toxicity of veratridine at 3 μM when applied in combination (150 $\pm$ 9% (n = 3) compared to 100% at 3 μM veratridine alone). ScTX is known to only prolong action potentials by slowing down the inactivation that occurs without modifying resting membrane potentials. It is likely that this difference reflects the difference of the mode of action of these two agents.

Involvement of the Na^+-Ca^{2+} Exchanger Activity in the Sustained Levels of $[Ca^{2+}]i$ in Cultured SCG Neurons in Response to Veratridine

The sustained increase in $[Ca^{2+}]i$ in response to veratridine shown in Fig. 3B suggests that voltage-dependent Ca^{2+} channels or the Na^+-Ca^{2+} exchanger or both is involved in this phenomenon. For this purpose, we have employed nifedipine, an inhibitor of voltage-dependent Ca^{2+} channels and benzamil, an inhibitor of Na^+-Ca^{2+} exchanger. Thus, sustained levels of $[Ca^{2+}]i$ of the neurons were measured following veratridine treatment for 1 h relative to the peak levels: 0.791 $\pm$ 0.027 (the mean $\pm$ SD; n = 16) for control, 0.548 $\pm$ 0.041 (n = 16) for benzamil (10 μM)-treated neurons, and 0.754 $\pm$ 0.063 (n = 16) for nifedipine (10 μM)-treated neurons. These results show that the sustained levels are significantly lower in neurons with benzamil, an inhibitor of the Na^+-Ca^{2+} exchanger compared to untreated control (Student's t-test, $p < 0.05$), but remain unaltered in neurons with nifedipine, an inhibitor of voltage-dependent Ca^{2+} channels. Furthermore, we have addressed the question as to whether or not the Na^+-Ca^{2+} exchanger functions in these neurons. The activity was evaluated by measuring an increase in $[Ca^{2+}]i$ by reversal of the Na^+-Ca^{2+} exchanger. The neurons loaded with fura-2 were incubated for 60 min with 50 μM ouabain, and their $[Ca^{2+}]i$ levels were measured in Ca^{2+}-free Na^+ HEPES saline or NMG^+ HEPES saline (Na^+ was replaced by N-methyl glucamine (NMG^+)), pH 7.2 at 32–34°C. The ratio ($F_{340nm}/F_{380\ nm}$) was monitored for 15 min following addition of 1 mM Ca^{2+}, and its increases were calculated for neurons in Na^+ or NMG^+(Na^+ free) saline. RATIO is defined as a ratio of the two values (ratio increase in NMG^+ saline/ratio increase in Na^+ saline). RATIO = 1 means that no exchanger activity is detectable. The activity was indeed detected (RATIO = 4.95 $\pm$ 1.35 (n = 46) compared to that in the presence of the inhibitor: 1.00 $\pm$ 0.51 (n = 36); $p < 0.001$). This result shows that the exchanger activity, but not voltage-dependent Ca^{2+} channels, is likely to be involved in maintaining the sustained levels of $[Ca^{2+}]i$ in response to veratridine at SCG neurons.

Veratridine Neurotoxicity was Dependent on Na^+ Influx Through Voltage-Dependent Na^+ Channels

Measurements of $[Na^+]i$ and $[Ca^{2+}]i$ in veratridine-treated SCG neurons suggest that veratridine neurotoxicity is essentially

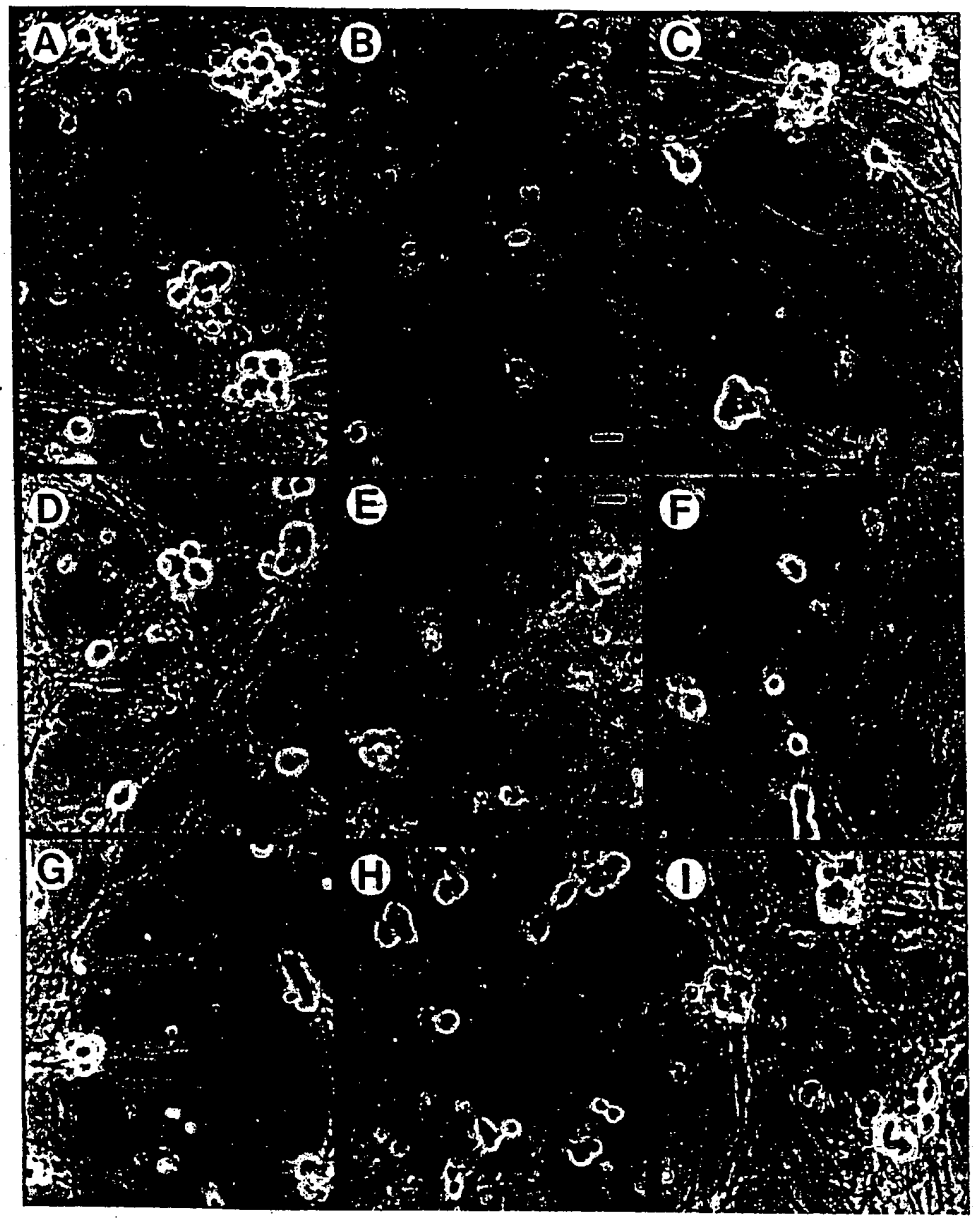

FIG. 1. Phase-contrast photomicrographs showing veratridine neurotoxicity in cultured superior cervical ganglion (SCG) neurons under various conditions. SCG neurons having grown in the presence of nerve growth factor (NGF) for 6–7 days were incubated for a further 24 h in the presence of NGF under various conditions and photographed. During this period, the neurons were exposed to 10 μM veratridine (B, C, E, F, H, I). Veratridine-treated neurons (B) became dark under phase-contrast microscope, and neurites were completely disintegrated compared to the control culture (A). The veratridine neurotoxicity was completely abolished by the presence of tetrodotoxin (TTX: 1 μM, C). Veratridine neurotoxicity was also examined in Ca^{2+}-free (E, F), or a low Na^+ (36 mM; H, I) medium in the presence of TTX (F, I) or in its absence (E, H). (D) and (G) show control neurons in the presence of NGF in Ca^{2+}-free medium or a low Na^+ medium, respectively. Scale bar: 25 μm.

due to either Na^+ influx, Ca^{2+} influx, or both. When SCG neurons were treated with 10 μM veratridine in a Ca^{2+}-free medium (Ca^{2+}-free MEM supplemented with 10% FBS dialyzed against Ca^{2+}-free MEM and 0.2 mM EGTA), veratridine neurotoxicity occurred as in normal medium (Fig. 1E, Fig. 4). In contrast, veratridine neurotoxicity was abolished in a low Na^+ medium (36 mM) in which Na^+ was replaced with N-methyl glucamine (Figs. 1H, 4, 5), suggesting that its neurotoxicity was due to Na^+ influx.

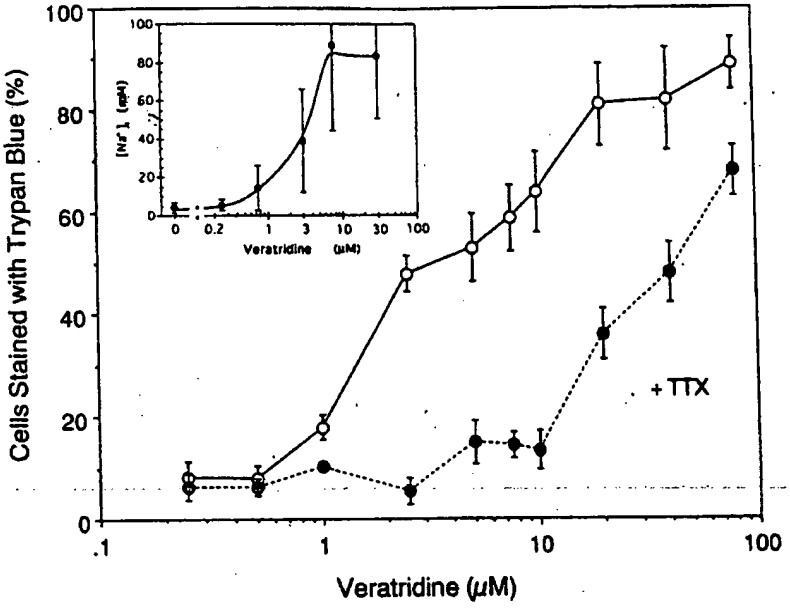

FIG. 2. Dose-response curve for veratridine neurotoxicity in sympathetic neurons *in vitro*. Cultured sympathetic neurons were exposed to various concentrations of veratridine (0.1–80 μM) for 24 h in the presence (●) or absence (○) of tetrodotoxin (TTX). Please note that protection of veratridine neurotoxicity by 1 μM TTX was lost at concentrations >20 μM. The inset indicates a dose-response curve showing the level of $[Na^+]i$ as a function of veratridine; Sympathetic neurons (1 week *in vitro*) loaded with SBFI-AM were exposed to 7.5 μM veratridine for 60 min, and then their intracellular Na^+ levels were measured as described. Mean ± SD.

but not to Ca^{2+} influx. Figure 5 shows a dose-response curve of veratridine neurotoxicity as a function of extracellular Na^+. Figure 1H also shows that the cell soma remained intact at 36 mM extracellular Na^+, but the neurites were still fragmented, suggesting that soma and neurites may have a different set-point for Na^+ neurotoxicity. TTX reversed veratridine neurotoxicity to both cell soma and neurites (Fig. 1H). Benzamil (25 μM) [16], acting as a potent Na^+-channel inhibitor at this concentration, abolished veratridine neurotoxicity, but the L-type Ca^{2+} channel blockers, nimodipine (2 μM) and nifedipine (4 μM), did not attenuate the toxicity (data not shown), being consistent with the fact that this blocker (nifedipine) did not attenuate the sustained levels of $[Ca^{2+}]i$ as described. These findings suggest that veratridine neurotoxicity to SCG neurons depends primarily on Na^+ influx via the activation of voltage-dependent Na^+ channels.

Buffering Action of BAPTA-AM on the Sustained Increase in $[Ca^{2+}]i$ Levels Did Not Attenuate Veratridine Neurotoxicity in SCG Neurons

BAPTA-AM has been used as an effective chelator of changes in $[Ca^{2+}]i$ levels. We have previously shown that prior treatment of SCG neurons with 10–50 μM BAPTA-AM resulted in impairment of membrane depolarization-induced increase in $[Ca^{2+}]i$ and elevated K^+-mediated rescue of these neurons from NGF deprivation-induced cell death [20,21]. SCG neurons in normal medium were treated with various concentrations of BAPTA-AM for 30 min, then veratridine was added and incubated for a further 10 h. The neurons treated with 50 μM BAPTA alone died upon longer incubation periods. Figure 6 shows that veratridine is toxic even in the presence of 50 μM BAPTA-AM. In the presence of 50 μM BAPTA, 70–80% of the sustained levels of $[Ca^{2+}]i$ were eliminated. Similarly, effects of chelation of intracellular Ca^{2+} with BAPTA-AM was also examined for the low Na^+ effect. Figure 6 shows that veratridine neurotoxicity was suppressed in the low Na^+ medium even in the presence of BAPTA-AM, although BAPTA treatment alone became slightly toxic under these conditions.

Elevated K^+ Abolished Veratridine Neurotoxicity in SCG Neurons

SCG neurons underwent shrinkage in response to veratridine under our conditions even though Na^+ overload occurred, suggesting the presence of a mechanism by which osmotic changes due to an overload of either Na^+ influx, Ca^{2+}, or both may be eventually compensated. Figure 5 shows that veratridine neurotoxicity was diminished in a dose-dependent manner as a function of extracellular K^+; and both cell soma and neurites were intact at 75 mM K^+. Measurements of $[Na^+]i$ levels revealed that the veratridine-induced increase in $[Na^+]i$ was completely suppressed under depolarizing conditions with 75 mM K^+; the level of $[Na^+]i$ under these conditions was 4.2 ± 2.0 mM (n = 28) even after treatment with 5 μM veratridine for 20 min. It is thus likely that the veratridine-induced influx of Na^+ or Ca^{2+} may occur in conjunction with the efflux of K^+ and possibly water. The inhibitors of K^+ channels, apamin (5 μM) and quinine (50–100 μM), were found to partially block veratridine neurotoxicity. For example, when the cells were treated with veratridine in the presence of 5 μM apamin for 24 h, 37.6 ± 4.0% (n = 3) of the cells were stained with trypan blue, whereas 67.0 ± 3.2% (n = 3) of them were dead in the absence of this blocker ($p < 0.05$). Figure 7 shows that Ca^{2+}-free

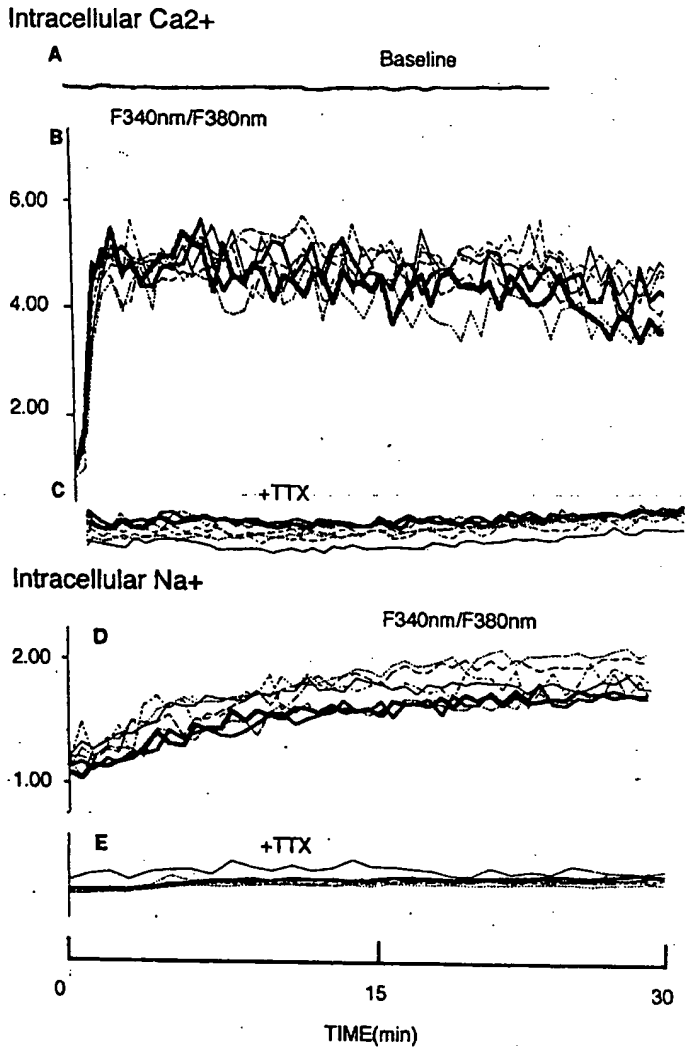

FIG. 3. Veratridine-induced increase in intracellular Ca^{2+} and Na^+ levels of sympathetic neurons. Sympathetic neurons (1week *in vitro*) loaded with fura-2-AM were exposed to 10μM veratridine and their intracellular Ca^{2+} levels were monitored (Trace A: baseline; Trace B: veratridine). F_{340nm}/F_{380nm} of 7 single neurons were monitored simultaneously for 30 min in response to veratridine (B). When tetrodotoxin (TTX; 1μM) was applied prior to veratridine treatment the sustained increase in $[Ca2^+]i$ was completely eliminated (Trace C). Other set of neurons loaded with sodium-binding benzofuran isophthalate-acetxymethyl ester (SBFI-AM) were exposed to 10 μM veratridine and their intracellular Na^+ levels were measured as well (Trace D: veratridine;Trace E: veratridine in the presence of TTX (1μM)).

elevated-K^+ medium or BAPTA-AM, an intracellular Ca^{2+} chelator, significantly impaired the toxicity-suppressing effect of elevated K^+, suggesting that cytoplasmic Ca^{2+} is essential for the depolarization-mediated rescue of veratridine-treated neurons from cell death.

Acidosis May Be a Major Pathway of Veratridine Neurotoxicity in SCG Neurons

As shown in Figs. 1 and 4, extracellular Ca^{2+} is not required for veratridine neurotoxicity, suggesting that the reverse operation of the Na^+/Ca^{2+} exchanger under depolarizing conditions is unlikely to play a major role in veratridine neurotoxicity in SCG neurons. A collapse of the sodium gradient is also likely to result in cytoplasmic acidification. We moved on to examine the role of the Na^+/H^+ exchanger in veratridine neurotoxicity. SCG neurons were labeled with BCECF-AM, a fluorescence pH indicator, and their cytoplasmic pH was monitored using a fluorescence imaging apparatus. Figure 8B shows the time course of pH decrease upon veratridine treatment. Upon treatment with veratridine for 6 h, the cytoplasmic pH remained low (pH 6.48 $\pm$ 0.73 (the mean $\pm$ SD.

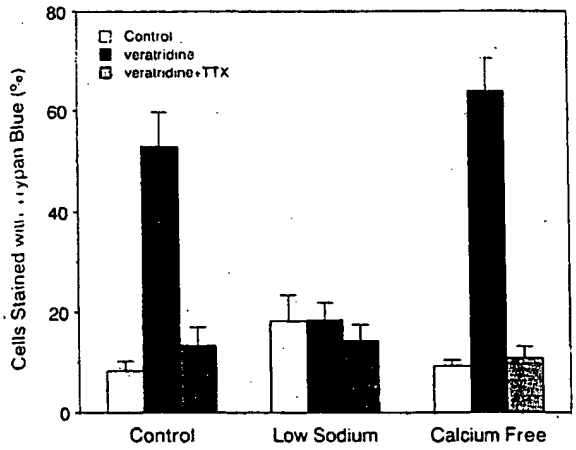

FIG. 4. Effects of extracellular Ca^{2+} and Na^+ on veratridine neurotoxicity. Sympathetic neurons (1 week *in vitro*) were treated with 10 μM veratridine for 24 h in normal (three left columns), a low Na^+ (36 mM; three middle columns), or Ca^{2+}-free (three right columns) medium in the presence of tetrodotoxin (TTX) (+TTX) or in its absence, and then neuronal survival was measured. Mean ± SEM (n = 3).

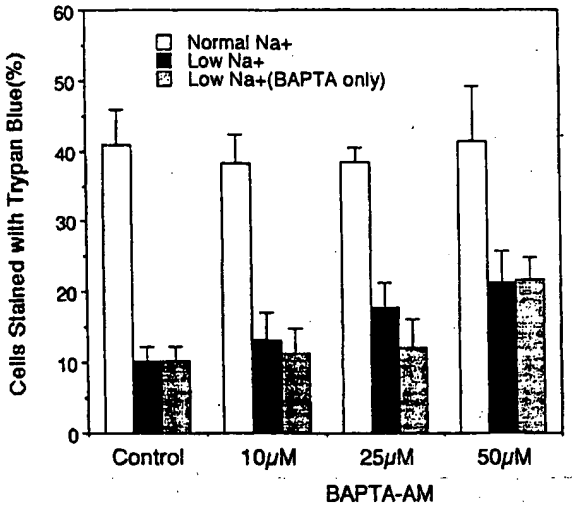

FIG. 6. Effects of the sustained increase in intracellular Ca^{2+} on veratridine neurotoxicity. Sympathetic neurons (1 week *in vitro*) were treated with 0, 10, 25, and 50 μM 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetate acetomethoxyester (BAPTA-AM) for 30 min prior to exposure to 10 μM veratridine for 10 h in normal medium (□). The neurons stained with trypan blue in the presence of 50 μM BAPTA alone for 10 h were 11 3.5% (n = 3). The neurons in a low Na^+ (36 mM) were also exposed to 0, 10, 25, and 50μM BAPTA-AM for 30 min, and then left untreated (■) or treated with 10 μM veratridine for 15 h (▨). Neuronal survival was then measured as described. Veratridine neurotoxicity in the low Na^+ medium was suppressed in the presence of BAPTA-AM, although BAPTA treatment alone became slightly toxic under these conditions. Mean ± SEM (n = 3).

n = 107) compared to the normal pH 7.00 ± 0.30 (n = 76; $p <$ 0.005)). A shown in Fig. 8C, EIPA suppressed the veratridine-induced decrease in pH. Under these conditions, EIPA (1 μM) had no effects on the veratridine-induced influx of Na^+ or Ca^{2+} (data not shown).

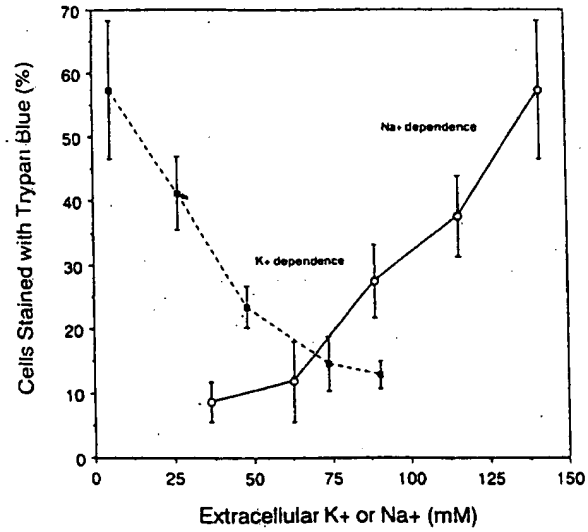

FIG. 5. Dose-response curves of extracellular K^+ and Na^+ for veratridine neurotoxicity in sympathetic neurons *in vitro*. Cultured sympathetic neurons were exposed to 10 μM veratridine for 24 h in medium containing various concentrations of Na^+ by being replaced with an equal amount of N-methyl-D-glucamine, and then neurotoxicity was measured. The effect of extracellular K^+ was also examined similarly by adding various conventrations of KCl to normal medium in the presence of veratridine. Mean ± SEM (n = 3).

In accordance with the results of the pH measurements, veratridine neurotoxicity was significantly attenuated by EIPA in a dose-dependent fashion (IC_{50} = 0.5 μM; Fig. 9). These findings suggest that Na^+ overload results in a mild cytoplasmic acidifica-

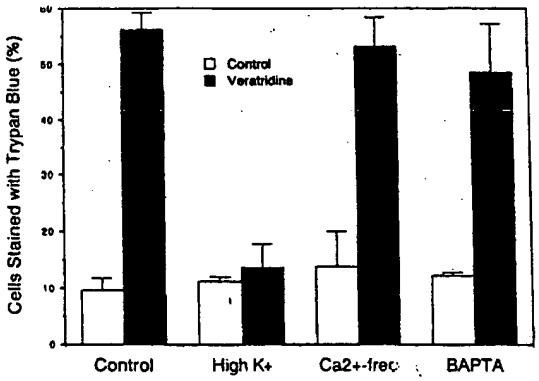

FIG. 7. Effects of Ca^{2+} on elevated-K^+ mediated suppression of veratridine-induced neuronal death. Cultured sympathetic neurons were exposed to 10 μM veratridine (filled columns) for 24 h in normal (control), an elevated K^+ (73 mM; high K^+), or Ca^{2+}-free (Ca^{2+}-free), or a medium containing 25 μM 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetate acetomethoxyester (BAPTA-AM), and then neurotoxicity was measured. Mean ± SEM (n = 3).

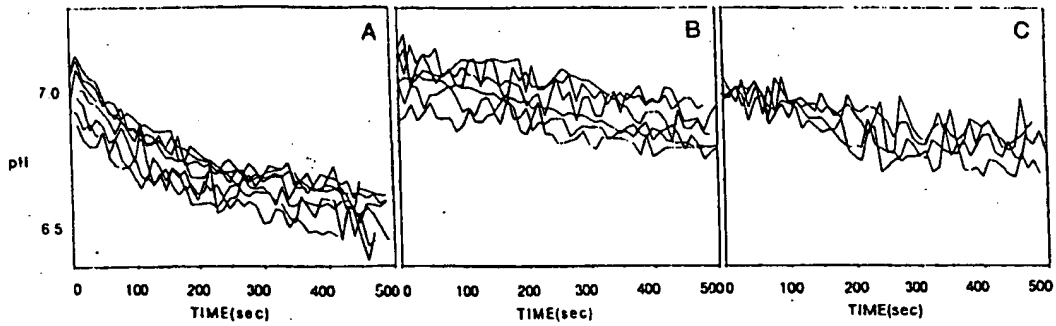

FIG. 8. Time course of pH changes in response to veratridine. Cultured sympathetic neurons were exposed to 10 μM veatridine in the absence (A) or presence (C) of 1 μM 5-N-ethyl-N-isopropyl-amiloride (EIPA), the specific inhibitor of the Na^+/H^+ exchanger. F_{340nm}/F_{380nm} of five single neurons were monitored simultaneously for 500 s. B represents the base line.

tion due to the Na^+/H^+ exchanger and plays a major role in the mechanism by which veratridine exerts neurotoxicity in these neurons.

Veratridine Exerts its Neurotoxicity Through Both Apoptotic and Necrotic Pathways

We have moved on to examine the mechanism of neuronal death caused by an excessive influx of Na^+ in SCG neurons. When veratridine-treated neurons were stained with bis-benzimide, 8.5 ± 2.5% (n = 3) of them exhibited fragmented nuclei, the classical hallmark of apoptosis, whereas 1.6 ± 0.4 % (n = 3) were positive in untreated cells (Fig. 10). Fragmented nuclei were also counted for these neurons undergoing cell death following NGF deprivation as a classical reference for apoptosis in these neurons. In these experiments, we only counted the number of fragmented nuclei, but veratridine caused a rapid alteration of the nuclei from normal to patch-like structures when stained with bis-benzimide 2 h after veratridine treatment, suggesting the ion influx also altered the chromatin structure of these neurons. We have also assessed the neurons with the TUNEL. In response to veratridine (10 μM for 15 h), 10.8 ± 1.5 % (n = 3) of the cells showed apoptotic nuclei, whereas only 2.66 ± 1.70 % (n = 3) of the cells were TUNEL-positive in control cells. Moreover, we have carried out combined bis-benzimide and EM examinations. The neurons undergoing chromatin fragmentation detected by bis-benzimide were identified and examined by electron microscopy. Figure 11 shows an example of one neuron undergoing cell death in the presence of 10

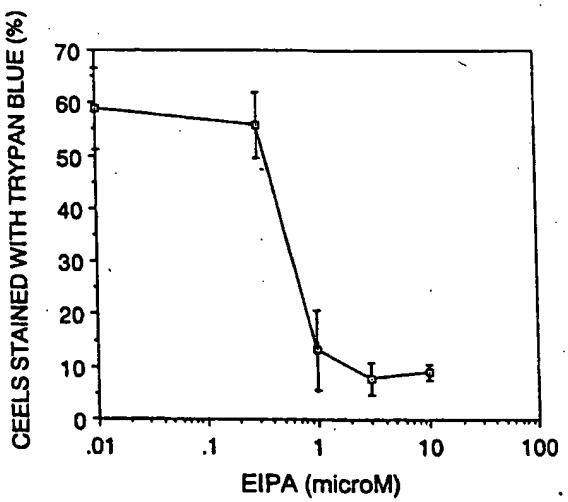

FIG. 9. Dose-response curve showing that 5-N-ethyl-N-isopropyl-amiloride (EIPA), the specific inhibitor of the Na^+/H^+ exchanger, suppresses veratridine neurotoxicity in sympathetic neurons *in vitro*. Cultured sympathetic neurons were exposed to various concentrations of EIPA (0.01–10 μM) for 24 h in the presence of 10 μM veratridine, and then neuronal survival was measured. Mean ± SEM (n = 3).

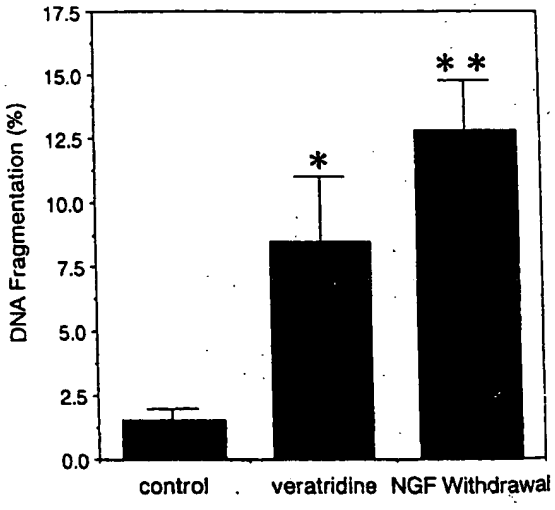

FIG. 10. Apoptotic nature of veratridine-induced cell death in cultured superior cervical ganglion (SCG) neurons. The neurons were treated with 10 μM veratridine for 15 h, and then fixed with 4% paraformaldehyde. Fragmented nuclei were counted for untreated and veratridine-treated neurons as described in the Materials and Methods. Bis-benzimide staining was also made for these neurons undergoing cell death following nerve growth factor (NGF) deprivation for 18 h as a classical model for apoptosis in these neurons. Student's t-test, $^*p < 0.05$; $^{**}p < 0.01$.

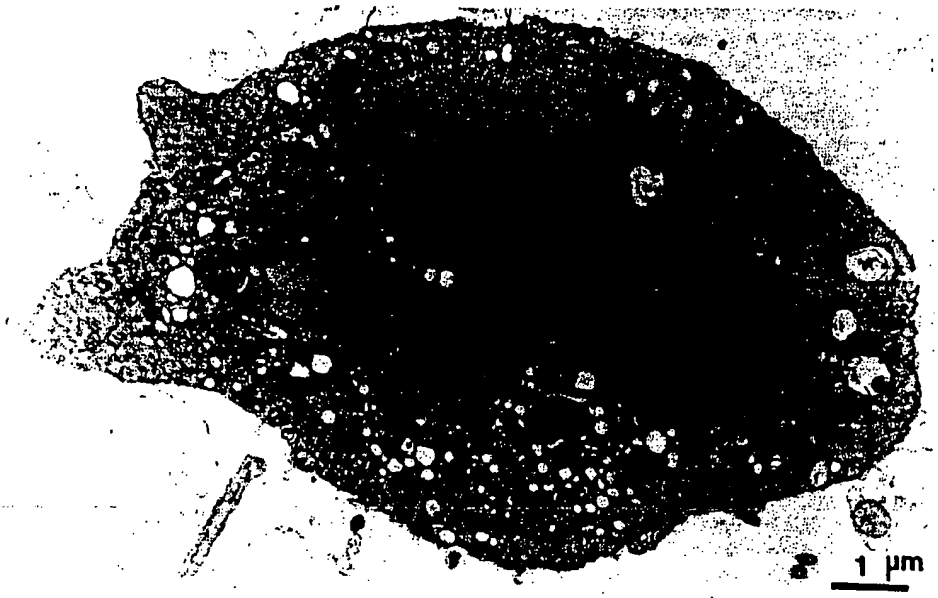

FIG. 11. Electron photomicrograph showing that apoptosis occurs in veratridine neurotoxicity in cultured superior cervical ganglion neurons. The bis-benzimide-positive neurons were identified by marking the area of plastic plates to which identified neurons were attached and photographed and were then post-fixed with 1% osmium tetra oxide and embedded in Epon 812. Identified regions were cut, stained, and examined. Please note that autophagic vacuoles and clear vesicles along with nuclear features of apoptosis are seen. Scale bar: 1 μm.

μM veratridine for 18 h (we have tested more than 10 cells). The cytoplasm of this neuron was dark and shrunken significantly with clear signs of nuclear condensation and fragmentation (Fig. 11). Moreover, this nuclear change is associated with extensive formation of clear vesicles and autophasic vacuoles in its cytoplasm, while mitochondria remained intact, demonstrating strong evidence for apoptosis (Fig. 11). These findings for the identified neurons confirmed that bis-benzimide positive neurons are in deed apoptotic based on EM criteria. These experiments unequivocally demonstrate that apoptotic pathway, although minor, occurred in SCG neurons in response to veratridine.

DISCUSSION

Veratridine, the activator of voltage-dependent Na^+ channels, is known to cause both Na^+ and Ca^{2+} influxes into the cytoplasm through Na^+ channels in neuroblastoma cells [18]. Veratridine neurotoxicity, however, was due to the influx of Na^+; a medium low in Na^+ completely abolished its neurotoxicity, while a Ca^{2+}-free medium did not attenuate its neurotoxicity. This suggests that in contrast to hippocampal neurons, the reverse operation of the Na^+/Ca^{2+} exchanger under depolarizing conditions is unlikely to play a major role in veratridine neurotoxicity in SCG neurons. Thus, the veratridine-mediated collapse of the sodium gradient may be likely to result in cytoplasmic acidification due to Na^+/H^+ exchangers as a possible mechanism of cell death. Indeed, there is evidence that the Na^+/H^+ exchanger has a pivotal role in regulating cytoplasmic pH in SCG neurons[37]. We have offered here evidence supporting this scenario by measuring cellular pH by fluorescent imaging, as well as by the use of its inhibitor EIPA, a derivative of amiloride [13].

Neurons regulate their cytoplasmic pH through a variety of ion transport mechanisms including Na^+/H^+ transporters, ATP-driven H^+ pumps, and several bicarbonate exchangers [1,17,39]. The Na^+/H^+ exchanger is inoperative at neutral pH, but has a high affinity for H at pH 6, so that any excess H^+ which is generated is extruded by the exchanger driven by an inward-directed Na^+ gradient under normal conditions. There is indeed evidence that the Na^+/H^+ exchangers are known to play a major role in regulating cytoplasmic pH in SCG neurons [37]. Under conditions of a normal electrochemical gradient, the Nernst equation calculates that complete inhibition of pH regulation will cause the pH to drop 1 pH unit. The cytoplasmic acidification observed in theses studies is below this theoretical prediction. However, it may be sufficient for disruption of the biosynthetic protein traffic pathway in the cells which utilizes the H^+ gradient between the cytoplasm and the transport organelle.

Growth factors are known to stimulate DNA synthesis and cell proliferation by altering cytoplasmic pH. Conversely, withdrawal of growth factors or interleukins result in cellular acidification, which is often associated with apoptosis [12,23]. It is postulated that cellular acidification may activate Ca^{2+}-independent DNases which yield fragmented DNA as detected by gel electrophoresis. DNA fragmentation is a hallmark of apoptosis. Alternatively, cellular acidification alters the phosphorylational states of cellular components, thus modifying the chromatin structure or ion pumps. It has been thus proposed that alteration in the set-point of the Na^+/H^+ antiport, possibly as a result of its dephosphorylation, is involved in cellular acidification in the apoptotic pathway. Although cytoplasmic acidification is not a prerequisite for cell death, it is often an early event of apoptosis [23].

Both *in situ* endolabeling and EM observations demonstrate that both apoptosis and necrosis occur upon treatment with veratridine in SCG neurons. In addition, there are neurons that exhibit a mixed form of cell death. This also happens in glutamate-induced

neuronal death in cortical neurons. Under conditions where low concentrations of N-methyl-D-aspartate are exposed to these neurons, only a mild and delayed form of cell death occurs predominantly characterized by apoptosis, while intense exposure of higher concentrations of glutamate causes necrotic cell damage characterized by acute swelling and lysis, thus forming a spectrum from necrosis and apoptosis [30].

The possibility that Na^+ overload may cause cell death via an alternate mechanism, however, remains undetermined. For example, there is evidence that Na^+ influx may alter the intracellular signaling system, including stimulation of the formation of inositol phosphates in neurons[14]. Thus, it is possible that a Na^+ influx increases the release of arachidoic acid and/or the production of oxidative products to damage veratridine-treated neurons, thus leading to cell death through this particular mechanism.

We have here offered evidence that veratridine, the voltage-dependent Na^+ channel activator, promotes both apoptotic and necrotic cell death in cultured sympathetic neurons. Veratridine neurotoxicity was due to the influx of Na^+; a low Na^+ medium completely abolished its neurotoxicity, while a Ca^{2+}-free medium did not attenuate its neurotoxicity. Furthermore, the buffering action of BAPTA on veratridine-induced increase in intracellular Ca^{2+} levels neither blocked veratridine neurotoxicity in normal medium, nor attenuated the low K^+ effect. Cytoplasmic pH measurements using a fluorescent pH indicator demonstrated that cellular acidification (from pH 7.0 to pH 6.5) occurred upon treatment with veratridine. Both veratridine-induced acidification and cell death were ameliorated by EIPA, the specific inhibitor of the Na^+/H^+ exchanger (IC_{50} = 0.5 μM) suggesting that Na^+ overload, independently of Ca^{2+}, plays a role in veratridine neurotoxicity in SCG neurons.

ACKNOWLEDGMENTS

This work was partly supported by a special Grant-in-Aid for the Promotion of Research and Education in Hokkaido University, the MESC Grant-in-Aid for Scientific Research on Priority Areas and the Program of Promotion of Fundamental Studies in Health Sciences.

REFERENCES

1. Agrawal, S. K.; Fehrings, M. G. Mechanisms of secondary injury to spinal cord axons in vitro: Role of Na^+, Na^+-K^+-ATPase, the Na^+/H^+ exchanger, and the Na^+/Ca^{2+} exchanger. J. Neurosci. 16: 545–552; 1996.
2. Ankarcrona, M.; Dypbukt, J. M.; Bonfoco, E.; Zhivotovsky, B.; Orrenius, S.; Lipton, S. A.; Nocotera, P. Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. Neuron 15:961–973; 1995.
3. Ashton, D.; Willems, R.; Marrannes, R.; Janssen, P. A. J. Extracellular ions during veratridine-induced neurotoxicity in hippocampal slices: Neuroprotective effects of flunaridine and tetrodotoxin. Brain Res. 528:212–22; 1990.
4. Bals, R.; Hack, N.; Jorgensen, O. S. Interactive effects involving different classes of excitatory amino acid receptors and the survival of cerebellar granule cells in culture. Int. J. Dev. Neurosci. 8:347–359; 1990.
5. Bhave, S. V.; Malhotra, R. K.; Wakade, T. D.; Wakade, A. R. Veratrin supports the in vitro survival of embryonic chick sympathetic neurons. Neurosci. Lett. 109:201–205; 1990.
6. Choi, D. W. Ischemia-induced neuronal apoptosis. Curr. Opin. Neurobiol. 6:667–672; 1996.
7. Collins, F.; Schmidt, M. F.; Guthrie, P. B.; Kater, S. B. Sustained increase in intracellular calcium promotes neuronal survival. J. Neurosci. 11:2582–2587; 1991.
8. Dargent, B.; Arsac, C.; Tricaud, N.; Couraud, F. Activation of voltage-dependent sodium channels in cultured cerebellar granule cells induces neurotoxicity that is not mediated by glutamate release. Neuroscience 73:209–216; 1996.
9. Erecika, M.; Dagani, F.; Nelson, D.; Deas, J.; Silver, I. A. Relations between intercellular ions and energy metabolism: A study with monensin in synaptosomes, neurons, and C6 glioma cells. J. Neurosci. 11:2410–2421; 1991.
10. Fiedman, J. E.; Haddad, G. G. Removal of extracellular sodium prevents anoxia-induced injury in freshly dissociated rat CA1 hippocampal neurons. Brain Res. 641:57–64; 1994.
11. Furber, S.; Oppenheim, R. W.; Prevette, D. Naturally-occurring neuron death in the ciliary ganglion of the chick embryo following removal of preganglionic input: Evidence for the role of afferents in ganglion cell survival. J. Neurosci. 7:1816–1832; 1987
12. Gottlieb, R. A.; Giesing, H. A.; Xhu, J. Y.; Engeler, R. L.; Babior, B. M. Cell acidification in apoptosis: Granulocyte colony-stimulating factor delays programmed cell death in neutrophils by up-regulating the vacuor H^+-ATPase. Proc. Natl. Acad. Sci. USA 92:5965–5968; 1995.
13. Gupta, S.; Cragoe, E. J. Jr; Deth, R. C. Influence of atrial natriuretic factor on 5-(N-ethyl-N-isopropyl)amiloride-sensitive $^{22}Na^+$ uptake in rabbit aorta. J. Pharmacol. Exp. Ther. 248:991–996; 1989.
14. Gusovsky, F.; Daly, J. W. Formation of second messengers in response to activation of ion channels in excitable cells. Cell. Mol. Neurobiol. 8:157–159; 1988.
15. Hall, D. H.; Gu, G.; Garcia-Anoveras, J.; Chalfie, M.; Driscoll, M. Neuropathology of degenerative cell death in caenorhabditis elegans. J. Neurosci. 17:1033–1045; 1997
16. Harootunian, A. T.; Kao, J. P. Y.; Eckert, B. K.; Tsien R. Y. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. J. Biol. Chem. 264:19458–19467; 1989.
17. Hoffmann, E. K.; Simonsen, L. O. Membrane mechanisms in volume and pH regulation in vertebrate cells. Physiol. Rev. 69:315–382; 1989.
18. Jacques, M.; Frelin, C.; Vigue, P.; Romey, G.; Parjari, M.; Lazdunski, M. Neurotoxins specific for the sodium channel stimulate calcium entry into neuroblastoma cells. Biochemistry 20:6219–6225; 1981.
19. Johnson, E. M. Jr; Deckwerth, T. L. Molecular mechnaisms of developmental neuronal death. Ann. Rev. Neurosci. 16:31–46; 1993.
20. Koike T.; Martin D. P.; Johnson E. M. Jr. Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death caused by trophic-factor deprivation: Evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. Proc. Natl. Acad. Sci. USA 86:6421–6425; 1989.
21. Koike, T.; Tanaka, S. Evidence that nerve growth factor dependence of sympathetic neurons for survival in vitro may be determined by cytoplasmic free Ca^{2+}. Proc. Natl. Acad. Sci. USA 88:3892–3896; 1991.
22. Lakics, V.; Sebestyen, M. G.; Erdo, S. L. Vinpoceptine is a highly potent neuroprotectant against veratridien-induced cell death on primary cultures of rat cerebral cortex. Neurosci. Lett. 185:127–130; 1995.
23. Li, J.; Easrman, A. Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification: The role of the Na^+/H^+-antiport. J. Biol. Chem. 270:3203–3211; 1995.
24. Maderdrut, J. L.; Oppenheim, R. W.; Prevette, D. Enhaneement of naturally occurring cell death in the sympathetic and parasympathetic ganglia of the chicken embryo following blockade of ganglionic transmission. Brain Res. 444:189–194; 1988.
25. May, G. R.; Rowand, W. S.; McCormack, R. G.; Sheridan, R. D. Neuroprotective profile of lifarizine in rat cerebrocortical neurons in culture. Br. J. Pharmacol. 114:1365–1370; 1995.
26. Malgoouris, C.; Daniel, M.; Doble, A. Neuroprotective effects of riluzole on N-methyl-D-aspartate or veratridine induced neurotoxicity in rat hippocampal slices. Neurosci. Lett. 177:95–99; 1994.
27. Lysko, P. G.; Webb, C. L.; Yue, T. L.; Gu, J. L.; Feuerstein, G. Neuroprotective effects of tetrodotoxin as a Na^+ channel modulator and in gerbile global brain ischemia. Stroke 25:2476–2482; 1994.
28. Pauwels, P. J.; Van Assouw, H. P.; Peeters, L.; Leysen, J. E. Neurotoxic action of veratridine in rat brain neuronal cultures: Mechanism of

neuroprotection by Ca^{++} antagonists nonselective for slow Ca^{++} Channels. J. Pharmacol. Exp. Ther. 255:1117–1122; 1990.
29. Pauwels, P. J.; Leysen, J. E.; Janssen, P. A. J. Ca^{++} and Na^{+} channels involved in neuronal cell death. Protection by flunarizine. Life Sci. 48:1881–1893; 1991.
30. Portera-Cailliau, C., Price, D. L.; Martin, L. J. Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis continuum. J. Comp. Neurol. 378:70–87; 1997.
1. Ramnath, R. R.; Strange, K.; Rosenberg, P. A. Neuronal injury evoked by depolarizing agents in rat cortical cultures. Neuroscience 51:931–939; 1992.
32. Shier, W. T.; DuBourdieu, D. J. Sodium- and calcium-dependent steps in the mechanism of neonatal rat cardiac myocyte killing by ionophores I. The sodium-carrying ionophore, monensin. Toxicol. Appl. Pharmacol. 116:38–46; 1992.
33. Schnider, A. F.; Olson, E. C.; Spitzer, N. C.; Montal, M. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J. Neurosci. 16:6125–6133; 1996.
34. Spitzer, N. C.; Gu, X.; Olson, E. Action potentials, calcium transients and the control of differentiation of excitable cells. Curr. Opin. Neurobiol. 4:70–77; 1994.
35. Tanaka, S.; Koike, T. Caffeine promotes survival of cultured sympathetic neurons deprived of nerve growth factor through a cAMP-dependent mechanism. Biochim. Biophys. Acta 1175:114–122; 1992.
36. Tanaka, S.; Koike, T. Veratridine delays apoptotic cell death induced by NGF deprivation through a Na^{+}-depenedent mechansiom in cultured rat sympathetic neurons. Int. J. Dev. Neurosci. 15:15–27; 1997.
37. Tolkovsky, A. M.; Richards, C. D. Na^{+}/H^{+} exchange is the major mechanism of pH regulation in cultured sympathetic neurons: Measurements in single cell bodies and neurites using fluorescent pH indicator. Neuroscience 22:1093–1102; 1987.
38. Tsien, R.; Pozzan, T. Measurement of cytosolic free Ca^{2+} with quin2. Meth. Enzymol. 172:230–262; 1989.
39. Xie, Y.; Dengler, K.; Zacharias, E.; Wilffert, B.; Tegtmeier, F. Effects of the sodium channel blocker tetrodotoxin on cellular ion homeostasis in rat brain subjected to complete ischemia. Brain Res. 652:216–224; 1994.